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Comparative Platelet Binding and Kinetic Studies with Normal and Variant Factor IXa Molecules*

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We have recently shown that thrombin-stimulated human platelets have specific, saturable receptors for factor IXa, occupancy of which promotes factor X activation (Ahmad, S. S., Rawala-Sheikh, R., and Walsh, P. N. (1989) *J. Biol. Chem.* 264: 3244–3251, 20012–20016; Rawala-Sheikh, R., Ahmad, S. S., and Walsh, P. N. (1990) *Biochemistry* 29, 2606–2611). To study the structural requirements for factor IXa binding to platelets, equilibrium binding studies and kinetic studies of factor X activation were carried out with normal factor IXa and with two variant proteins: factor $IXa_{Alabama}$ ($FIXa_{AL}$; $Asp^{47} \rightarrow Gly$ substitution) and factor $IXa_{Chapel\ Hill}$ ($FIXa_{CH}$; $Arg^{145} \rightarrow His$ substitution). In the absence of factors VIIIa and X, there were 331 binding sites/platelet for $FIXa_{CH}$ ($K_{d\ app}$ = 2.8 nM), and 540 sites/platelet for $FIXa_{AL}$ ($K_{d\ app}$ = 3.2 nM), compared with 540 sites/platelet ($K_{d\ app}$ = 2.3 nM) for normal factor IXa. The addition of factors VIIIa and X, both at saturating concentrations, had no effect on the number of binding sites for either normal or variant factor IXa, resulted in a decrease in the K_d for normal factor IXa to 0.67 nM, resulted in a suboptimal decrease in K_d for $FIXa_{AL}$ (1.4 nM), and had no effect on the K_d for $FIXa_{CH}$. Kinetic studies of factor X activation at variable factor IXa concentration confirmed these values of K_d in the presence of factors VIIIa and X. Determination of rates of factor X activation at variable substrate concentrations yielded normal values of catalytic efficiency (k_{cat}/K_m) for the variant proteins, thereby indicating that the abnormally low rates of factor X activation obtained were a consequence of the low affinity binding of $FIXa_{AL}$ and $FIXa_{CH}$ to thrombin-activated platelets in the presence of factors VIIIa and X. These studies suggest that the presence of Asp^{47} and the cleavage of factor IX at Arg^{145}-Ala^{146} are important structural features required for specific, high affinity factor IXa binding to platelets in the presence of factors VIIIa and X.

Activated human platelets promote the activation of factor X by factor IXa (1, 2). Previous studies from our laboratory, aimed at elucidating the mechanisms by which platelets and factor VIII contribute to this coagulation reaction, have shown that thrombin-stimulated human platelets have specific, saturable binding sites for factor IXa and that the presence of factor VIII and factor X increases the binding affinity 5-fold (3). We have also shown that platelet receptor occupancy with factor IXa is closely correlated with rates of factor X activation (4, 5).

To study the structural requirements for factor IXa binding to platelets, we have now carried out detailed comparative platelet binding and kinetic studies with normal and variant factor IXa molecules. One of these proteins, factor $IX_{Alabama}$ (factor IX_{AL}),[1] can be activated by factor XIa in the presence of calcium ions to a factor IXa_β form with about 10% of the clotting activity of the normal factor IXa_β (6, 7). An adenine to guanine transition in the first nucleotide of exon d causes the substitution of a glycine codon (GGT) for the normal aspartic acid codon (GAT). This point mutation results in a single amino acid substitution at residue 47 of the zymogen in the first epidermal growth factor-like domain of factor IX_{AL} (8). The factor IX defects previously reported in the first epidermal growth factor-like domain are mostly associated with mild hemophilia B. In addition to factor IXa_{AL}, these include factor $IX_{London7}$ ($Pro^{55} \rightarrow$ Ala, 10% of normal activity) (9), factor IX_{Durham} ($Gly^{60} \rightarrow$ Ser, 14% of normal activity) (10), factor $IX_{London6}$ (β-OH $Asp^{64} \rightarrow$ Gly, 8% of normal activity), and factor $IX_{New\ London}$ ($Glu^{50} \rightarrow$ Pro, <1% of normal activity) (11).

The second variant protein we have studied is factor $IX_{Chapel\ Hill}$ (factor IX_{CH}). The molecular defect in factor IX_{CH} is the substitution of histidine for arginine at position 145 (12–14). This is the first cleavage site in the normal pathway of factor IX activation. Thus, factor IX_{CH} is not activated normally either by factor XIa or by factor VIIa-tissue factor. Only the Arg^{180}-Val^{181} bond is cleaved giving rise to factor IXa_α, in which the activation peptide remains covalently attached to the light chain. Factor $IXa\alpha_{CH}$ has 20% of the clotting activity of normal factor IXa_β. Mutations in certain abnormal factor IX proteins have been demonstrated to cause abnormally slow or incomplete activation of factor IX by factor XIa. These include defects such as that in factor IX_{Hilo} in which cleavage of the Arg^{180}-Val^{181} bond is prevented by substitution of glutamine for arginine at position 180. The dysfunction of factor $IX_{New\ London}$ has also been attributed to

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[1] The abbreviations used are: factor IX_{AL}, factor $IX_{Alabama}$; factor IX_{CH}, factor $IX_{Chapel\ Hill}$; factor IX_N, normal factor IX; PPACK, D-phenylalanyl-prolyl-arginyl chloromethyl ketone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

defective cleavage at the Arg^{180}-Val^{181} bond (11).

The lipid binding and kinetic properties of normal factor IXa (factor IXa_N) have been examined and compared in detail with the two variant proteins, namely factor IX_{AL} and factor IX_{CH} (15). In this paper we have carried out detailed studies to compare the platelet binding and kinetic studies of these abnormal variant factor IXa molecules with factor IXa_N.

EXPERIMENTAL PROCEDURES

Materials—D-Phenyl-alanyl-prolyl-arginyl chloromethyl ketone (PPACK) was purchased from Behring Diagnostics. The chromogenic substrate S2337 (Bz-Ile-Glu-(γ-piperidyl)-Gly-Arg-*p*-nitroanilide) was purchased from AB Kabi Diagnostica. Other materials were the same as reported previously (3–5).

Proteins—Details of the purification, assay, and characterization of human coagulation proteins, including factor IX, factor IXa, factor VIII, factor X, and α-thrombin, were previously published (3). The conditions used for activation of factor VIII were identical with those previously published (3–5). The variant factor IX molecules were isolated from human plasma as described previously (15). Both the normal and variant factor IX molecules were radiolabeled with ^{125}I by the Iodo-Gen method as previously described (3), and specific radioactivities of all proteins were in the range of $2.0–2.5 \times 10^6$ cpm/μg. Activation of purified factor IX_N, factor IX_{AL}, and factor IX_{CH} by purified human factor XIa were carried out as previously described (3). Autoradiograms of normal and variant factor IX and factor IXa were developed to provide structural characterization of ^{125}I-labeled proteins. Both ^{125}I-labeled factor IX_{AL} and factor IX_{CH} appeared as single bands at M_r = 57,000 (Fig. 1, *lanes 2* and *3*) and were indistinguishable from factor IX_N (Fig. 1, *lane 1*). Similar results were obtained with the zymogens after reduction (Fig. 1, *lanes 4, 5,* and *6*). Both factor IXa_N (Fig. 1, *lane 7*) and factor IXa_{AL} (*lane 8*) migrated nonreduced at an apparent M_r of ~45,000, whereas factor IXa_{CH} (*lane 9*) remained at a M_r of 57,000 since the activation peptide remained covalently attached to the light chain. After reduction, the ^{125}I-labeled factor IXa_{AL} migrated as two polypeptides of M_r = 27,000 and 17,000 (Fig. 1, *lane 11*), as did factor IXa_N (Fig. 1, *lane 10*), whereas ^{125}I-labeled factor IXa_{CH} appeared under reducing conditions (Fig. 1, *lane 12*) as two bands, in addition to a small quantity (8.9% by scanning of the autoradiogram), as previously reported (13) of the original zymogen. One of the two major bands migrated with a mobility identical with that of the heavy chain band from factor IX_N, whereas the other band had an apparent M_r of 45,000 which corresponds to a cleavage product consisting of the light chain and the activation peptide similar to that found on cleavage of either factor IX_N or factor IX_{CH} by Russell's viper venom (13, 16). The clotting activities of activated factor IX_{AL} and factor IX_{CH} were 15% and 18%, respectively, of the clotting activity of factor IXa_N. Protein concentrations were determined by the Bio-Rad dye binding assay according to instructions provided by the manufacturer. Polyacrylamide slab gel electrophoresis in $NaDodSO_4$ was carried out according to the procedure of Laemmli (17).

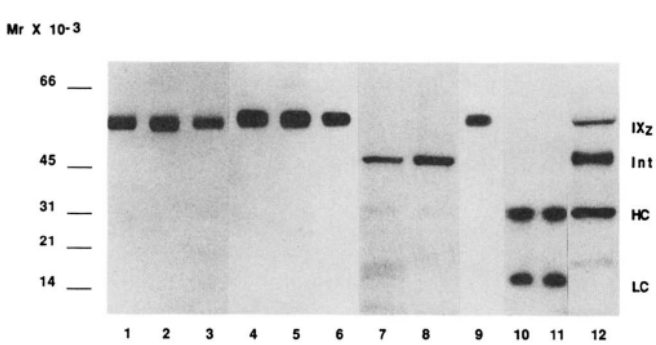

FIG. 1. **Autoradiogram of normal and variant ^{125}I-factor IX and factor IXa molecules.** Autoradiograms are shown of 13% polyacrylamide gel electrophoretograms in sodium dodecyl sulfate of ^{125}I-labeled factor IX_N, factor IX_{AL}, and factor IX_{CH} (nonreduced, *lanes 1, 2,* and *3*; and reduced, *lanes 4, 5,* and *6*, respectively) and factor IXa_N, factor IXa_{AL}, and factor IXa_{CH} (nonreduced, *lanes 7, 8,* and *9* and reduced, *lanes 10, 11,* and *12*, respectively). Abbreviations are as follows, showing positions of polypeptide chains in the gel: *IX_Z*, zymogen; *Int*, intermediate band consisting of light chain and activation peptide; *HC*, heavy chain; *LC*, light chain.

Binding Experiments—In a typical binding experiment, gel-filtered platelets ($3–4 \times 10^8$/ml) in calcium-free HEPES Tyrode's buffer, pH 7.4, were incubated at 37 °C in a 1.5-ml Eppendorf plastic centrifuge tube with mixtures of unlabeled and radiolabeled factor IXa, calcium chloride, platelet stimuli, and other proteins. Platelets were separated from unbound proteins as previously described (3). The data were analyzed and the number of binding sites and dissociation constants (K_d) were calculated from the means of six independent determinations, each done in duplicate, as previously described (3) using a Mac Plus Computer and the LIGAND Program as modified by McPherson (22).

Measurements of Rates of Factor Xa Formation—The activation of factor X by normal and variant factor IXa was carried out at 37 °C in the presence of thrombin-stimulated gel-filtered platelets, factor VIIIa, and calcium chloride as described previously (3–5). The details of experimental conditions and concentrations of reactants are given under "Results" and in the figure legends.

Calculations of Kinetic Constants—The derivation of kinetic constants of factor X activation by factor IXa was based on a one-enzyme, one-substrate model. The Michaelis constant (K_m) and the maximum velocity (V_{max}) were calculated from the mean ± S.E. of five independent determinations each done in duplicate of factor X activation rates at variable factor X concentrations as described previously (5). Values of dissociation constant (K_d) were obtained from experiments in which rates of factor X activation were determined at variable factor IXa concentrations as previously described (4). The values of turnover numbers (k_{cat}) were calculated by dividing V_{max} values either by the total factor IXa concentration or by the amount of enzyme (factor IXa) bound under the conditions of the experiment. This latter value was obtained from the equation:

$$\text{Amount bound} = \frac{B_{max} \cdot E}{K_d + E}$$

where B_{max} is the maximum amount of factor IXa bound or the total receptor concentration; E, total factor IXa concentration; and K_d, dissociation constant. The details of this calculation are provided in previous papers (4, 18).

RESULTS

Specific Binding of ^{125}I-Labeled Factor IXa_N, Factor IXa_{AL}, and Factor IXa_{CH} to Thrombin-activated Normal Human Platelets—In the present work, we have compared binding of ^{125}I-labeled factor IXa with the binding of factor IXa_{AL} and factor IXa_{CH} to normal human platelets. Scatchard analysis of the binding data (Fig. 2) gave straight lines indicating the presence of a single class of binding sites for both the normal and variant factor IXa molecules both in the presence and absence of factor VIIIa and factor X. The affinity and stoichiometry of binding for these ligands under both experimental conditions was determined in six separate experiments, the means (±S.E.) of which are given in Table I. In addition, the stoichiometry and affinity of factor IX binding was determined as previously reported (3), and the results were recorded in Table I. In the absence of factor VIIIa and factor X, there were 331 binding sites/platelet for factor IXa_{CH} ($K_{d\ app}$ = 2.8 nM) and 540 sites/platelet for factor IXa_{AL} ($K_{d\ app}$ = 3.2 nM), compared with 540 sites/platelet ($K_{d\ app}$ = 2.5 nM) for factor IXa_N, and 306 sites/platelet ($K_{d\ app}$ = 2.6 nM) for factor IX_N. The addition of factor VIIIa and factor X, both at saturating concentrations, had no effect on the number of binding sites for either normal or variant factor IXa molecules or for factor IX_N, resulted in a decrease in the K_d for factor IXa_N to 0.67 nM, resulted in a suboptimal decrease in K_d for factor IXa_{AL} (1.4 nM), and had no effect on the K_d for either factor IXa_{CH} or factor IX_N. The number of binding sites for factor IXa_{AL} was not significantly different from that for factor IXa_N. The number of binding sites for factor IXa_{CH} was significantly lower than that for factor IXa_N ($p < 0.01$) and was not significantly different from that for factor IX_N.

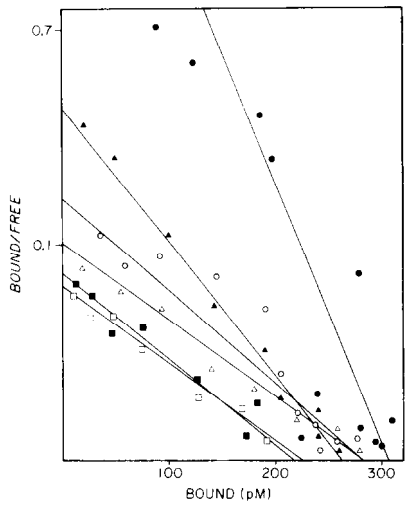

FIG. 2. **Scatchard analysis of the specific binding of ^{125}I-factor IXa$_N$, ^{125}I-factor IXa$_{AL}$, and ^{125}I-factor IXa$_{CH}$ to thrombin-stimulated normal human platelets in the absence and presence of factor VIII and factor X.** Gel-filtered platelets (3.5×10^8/ml) were incubated at 37 °C with human α-thrombin (0.1 unit/ml), $CaCl_2$ (5 mM), and ^{125}I-factor IXa$_N$, factor IXa$_{AL}$, or factor IXa$_{CH}$ in the presence or absence of thrombin-activated factor VIII (5 units/ml) and factor X (1.5 μM). Binding was determined as detailed under "Materials and Methods." Nonspecific binding was determined in the presence of excess unlabeled factor IXa (0.44 μM; 25 μg/ml) and was subtracted from total binding to obtain specific binding. The results shown represent a Scatchard plot of specific binding data for factor IXa$_N$ in the absence (○) and presence (●) of factor VIIIa and factor X compared with specific binding data for either factor IXa$_{AL}$ (△, ▲) or factor IXa$_{CH}$ (□, ■) in the absence (△, □) or presence (▲, ■) of factor VIIIa and factor X. The plotted results represent mean values from six experiments with normal and variant factor IXa molecules, each done in duplicate.

TABLE I

Binding constants for normal and variant factor IXa molecules

Ligand	Factor VIII	Number of sites per platelet (±S.E.)	K_d	
			Equilibrium	Kinetic
			nM	
Factor IXa$_N$	Absent	540 ± 68	2.3 ± 0.39	
	Present	590 ± 60	0.67 ± 0.05	0.61 ± 0.02
Factor IXa$_{AL}$	Absent	540 ± 52	3.2 ± 0.41	
	Present	506 ± 47	1.4 ± 0.09	1.4 ± 0.10
Factor IXa$_{CH}$	Absent	331 ± 44	2.8 ± 0.45	
	Present	310 ± 45	3.0 ± 0.51	2.0 ± 0.06
Factor IX$_N$	Absent	306 ± 57	2.6 ± 0.25	
	Present	316 ± 50	2.4 ± 0.30	

Kinetic Studies of Normal and Variant Factor IXa—In this study we also determined the apparent K_d for normal and variant factor IXa binding to platelets by kinetic studies of factor Xa formation in the presence of saturating concentrations of factor X and factor VIIIa (Fig. 3). The kinetic approach gave similar results to the binding studies (3, 4), and its use is justified in our previous studies (3–5). The apparent K_d was determined as 0.61 nM for factor IXa$_N$, 1.4 nM for FIXa$_{AL}$ and 2.0 nM for FIXa$_{CH}$ (Fig. 3 and Table I).

We also determined the kinetic parameters for factor X activation by normal and variant factor IXa molecules in the presence of thrombin-stimulated platelets and factor VIIIa (Fig. 4). Studies were carried out at a factor IXa concentration of 0.01 nM, well below the apparent dissociation constant for binding of factor IXa to platelets. The values of K_m, V_{max}, k_{cat}, and catalytic efficiency (k_{cat}/K_m) for factor IXa$_N$, factor IXa$_{AL}$ and factor IXa$_{CH}$ obtained at saturating concentrations of factor VIIIa are summarized in Table II. Catalytic efficiency can be assessed in two ways: 1) as a function of the total amount of factor IXa added, or 2) as a function of the amount of factor IXa bound to the platelet. If catalytic efficiency is assessed as a function of the amount of factor IXa *bound*, there is essentially no difference in the rate at which factor X is activated (the last column of Table II). So the decrease in the rate at which a given amount of factor IXa$_{AL}$ or factor IXa$_{CH}$ can catalyze factor X activation is solely due to the reduced affinity of these two proteins for the platelet-factor VIIIa-factor X complex.

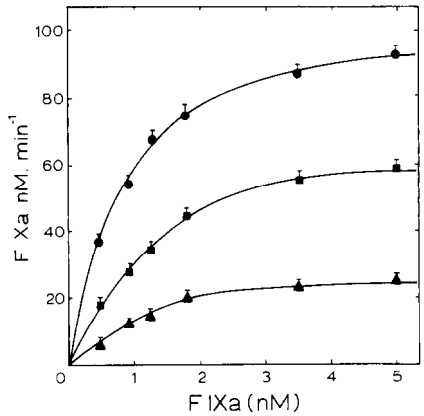

FIG. 3. **Rates of factor Xa formation by normal and variant factor IXa in the presence of thrombin-activated platelets and factor VIIIa.** The rates of activation of human factor X by varying concentrations (0.17–5.0 nM) of factor IXa were determined in the presence of 5×10^7/ml thrombin-activated platelets at 37 °C in a reaction volume of 100 μl containing 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 1.5 μM factor X, 5 units/ml of factor VIIIa, and 0.5 mg/ml human serum albumin. Platelets were stimulated with 0.1 unit/ml thrombin in the presence of $CaCl_2$ (5 mM), and factor IXa was preincubated with platelets for 10 min at 37 °C. Excess thrombin was neutralized with 50 nM PPACK before the addition of factor VIIIa and performance of the assay. For experimental details see "Experimental Procedures." The plotted results for factor IXa$_N$ (●), factor IXa$_{AL}$ (▲), and factor IXa$_{CH}$ (■) are the mean ± S.E. of duplicate observations from five separate experiments.

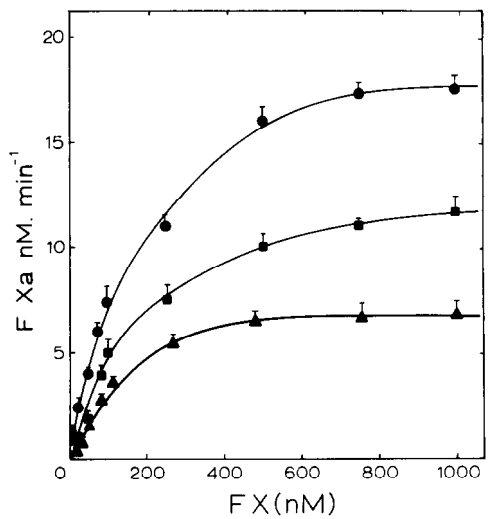

FIG. 4. **Factor X activation by normal and variant factor IXa.** Initial rates of factor X activation (mean ± S.E.) were determined as described under "Experimental Procedures" at various concentrations of factor X as indicated in the *graph*. The experiment was carried out essentially as described in the legend to Fig. 3 except that factor X concentration was varied and the concentration of normal or variant factor IXa was 10 pM. The plotted results for factor IXa$_N$ (●), factor IXa$_{AL}$ (▲), and factor IXa$_{CH}$ (■) are the mean ± S.E. of duplicate observations from five separate experiments.

TABLE II

Normal and variant factor IXa catalyzed factor X activation: kinetic analysis

	K_m	V_{max}	$k_{cat}{}^a$	$k_{cat}{}^b$	$k_{cat}{}^a/K_m$	$k_{cat}{}^b/K_m$
	μM	$nM \cdot min^{-1}$	min^{-1}		$\mu M^{-1} \cdot min^{-1}$	
Factor IXa_N	0.10	18.7	1,870	2,671	18,700	26,714
Factor IXa_{AL}	0.08	7.4	740	2,633	9,250	32,913
Factor IXa_{CH}	0.18	12.5	1,250	6,621	6,944	36,783

[a] k_{cat} expressed as moles of factor Xa formed per min per mol of total added factor IXa.

[b] k_{cat} expressed as moles of factor Xa formed per min per mol of total platelet-bound factor IXa.

DISCUSSION

The purpose of the studies reported here was to begin an analysis of the structural features of the factor IXa molecule that are important for factor IXa binding to platelets and for the assembly of the factor X activating complex on the platelet surface. Previously we have shown that factor IXa binds reversibly to 500–600 sites per platelet and that platelet activation and the presence of calcium ions are required for this interaction (3). The dissociation constant (K_d) for factor IXa binding to activated platelets is ~2.5 nM in the absence of factor VIIIa and factor X and ~0.5 nM in the presence of these proteins at saturating concentrations (3). Similar findings have been reported for bovine aortic endothelial cells by Stern *et al.* (19). We have also shown that zymogen factor IX binds to 250–300 sites per activated platelet in the presence of calcium ions with a K_d of ~2.5 nM either in the presence or absence of factors VIIIa and X and that factor IX competes with factor IXa for about one-half its low affinity sites in the presence of factors VIIIa and X (3). This suggests that the zymogen contains a domain important for binding of the enzyme to its platelet receptor and that the conversion of factor IX to factor IXa involves either a conformational alteration or the exposure of domains in factor IXa that allow it to interact with twice the number of platelet receptors as factor IX and also allow it to interact with factors VIIIa and X. We have further demonstrated that factor IXa binding to its high affinity site is closely correlated with rate enhancements of factor X activation by activated platelets (4), which can decrease the K_m for factor X activation and can permit factor VIIIa to increase the k_{cat} with a consequent increase of catalytic efficiency (k_{cat}/K_m) of (17.4×10^6)-fold (5). Finally, we have studied the role of the active site of factor IXa in the binding of the enzyme to platelets by examining the interaction with platelets of factor IXa active site inhibited with dansyl-L-glutamyl-glycyl-L-arginyl chloromethyl ketone (DEGR-CK). Since DEGR-factor IXa was shown to be a competitive inhibitor both of factor IXa binding and of factor X activation, with a K_i almost identical with the K_d for factor IXa binding, we concluded that the active site of factor IXa is not involved in binding to the high affinity site in the presence of factor VIIIa and factor X (4).

To determine the structural features of factor IXa that are required for interaction with platelet receptors and for assembly of the factor X activating complex, we studied the binding of factor IXa_{AL} and factor IXa_{CH} to activated platelets. It has previously been suggested (16, 20) that the abnormal coagulant activities of these two proteins could be a consequence of deficient binding to charged membrane surfaces. However, it was subsequently shown (15) that the abnormal rates of factor X activation observed with both factor IXa_{AL} and factor IXa_{CH} in the presence of small, unilamellar vesicles composed of 30% phosphatidylserine and 70% phosphatidylcholine were not a consequence of abnormal binding. Thus, the zymogen and activated forms of factor IX_N, factor IX_{AL}, and factor IX_{CH} were shown to bind with similar affinities to small, unilamellar vesicles as determined by 90° light scattering (15). It was therefore concluded that the normal function of factor IXa must entail interactions between the light and heavy chains on the phospholipid surface.

The present studies addressed the structural requirements for binding of factor IXa to what must be presumed the physiologic locus of factor X activation, *i.e.* the platelet membrane. Factor IXa_{AL} was shown to bind with normal affinity (K_d = 3.2 nM) to a normal number (540) of sites per platelet in the absence of factors VIIIa and X. This implies that the aspartic acid residue at position 47 in the normal protein (and mutated to a glycine in factor IX_{AL}) is not required for normal binding of factor IXa to its platelet receptor. This demonstration of a normal affinity of factor IXa_{AL} binding to platelet membranes in the absence of factors VIII and X confirms the results of Jones *et al.* (15) showing normal binding of factor IXa_{AL} to phospholipid membranes. However, in the presence of factors VIIIa and X, the binding affinity of factor IXa_{AL} was reduced (K_d = 1.4 nM) as determined either by equilibrium binding or by kinetic determinations of rates of factor X activation (Table I). This result is not in conflict with those of Jones *et al.* (15) since their phospholipid binding studies were carried out only in the absence of factors VIII and X.

The results of our kinetic analysis of factor X activation by factor IXa_{AL} (Table II) help to clarify the interpretation of our binding studies and are also in general agreement with the previous factor X activation studies of Jones *et al.* (15). Thus, we found the K_m for factor X activation by factor IXa_{AL} to be normal, suggesting that factor X binds with normal affinity to the factor IXa_{AL}-factor VIIIa-platelet membrane complex. The V_{max} (7.4 $nM \cdot min^{-1}$) with factor IXa_{AL} was 40% of normal, *i.e.* when compared with normal factor IXa (18.7 $nM \cdot min^{-1}$), a result consistent with the reduction in relative rate of factor X activation by factor IXa_{AL} (43% of normal) obtained by Jones *et al.* (15) with phospholipids. In the present study, however, since we carried out both equilibrium binding studies in parallel with factor X activation studies, we were able to calculate true catalytic constants (k_{cat}) expressing the maximal rate of factor Xa formation as a function of the amount of factor IXa bound (Table II). The result of this analysis demonstrates a normal turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) for factor IXa_{AL} compared with factor IXa_N, indicating that the defect in factor X activation by factor IXa_{AL} is entirely a consequence of its decreased affinity for platelet receptors in the presence of factor VIIIa. Therefore, although the interpretation of the molecular basis for the defect is open to further study, it would appear that the $Asp^{47} \rightarrow Gly$ mutation in factor IX_{AL} results in a decreased rate of factor X activation solely as a consequence of decreased affinity of the mutant enzyme for its platelet receptor in the presence of factor VIIIa and factor X.

A recent paper published by McCord *et al.* (21) reporting studies with factor IX_{AL} provides evidence for a conformational change in factor IX due to high affinity calcium binding in the first epidermal growth factor domain. These authors found that although factor IX_{AL} binds calcium ions normally to a high affinity site in the first epidermal growth factor domain, the variant enzyme fails to undergo a calcium-induced conformational change that occurs in normal factor IXa, thereby permitting it to interact properly with factor VIIIa and factor X. Thus, whereas factor IX_{AL} was activated normally by factor XIa and factor IXa_{AL} had 52–60% of normal activity in a calcium/phospholipid vesicle system, the addition of factor VIIIa decreased the relative rate of factor

X activation by factor IXa_{AL} to 18–19% of normal. These observations are consistent with our suggestion that the defect in factor IXa_{AL} is a consequence of its failure to bind with normal affinity to membranes in the presence of factor VIIIa.

Our studies with factor IXa_{CH} show that this variant enzyme binds to platelets in a manner indistinguishable from normal zymogen factor IX (Table I). Thus, the number of binding sites per activated platelet appears to be similar to that for normal factor IX and about half the number for normal factor IXa. Moreover, the affinity of binding, which is 5-fold enhanced for normal factor IXa in the presence of factors VIIIa and X, is unaffected by factors VIIIa and X in the case of factor IXa_{CH}. This very interesting result suggests that cleavage of factor IX at Arg^{145}-Ala^{146} (defective in factor IX_{CH} because of the $Arg^{145} \rightarrow$ His substitution), as well as at Arg^{180}-Val^{181}, with consequent formation of an activation peptide, is required for binding to the normal complement of receptors. In attempting to explain why the number of binding sites for factor IXa is almost exactly double that for zymogen factor IX, it is tempting to speculate that the receptor is bivalent (possibly homodimeric) and can accommodate two factor IXa molecules but only one factor IX molecule. It is possible that the presence of the heavily glycosylated activation peptide region of factor IX prevents access of a second factor IX molecule to the receptor complex, whereas formation of the activation peptide or its release from covalent attachment allows the resultant factor IXa access to both binding sites on the putative dimeric receptor. The fact that the affinity of factor IXa_{CH} for platelets is unaffected by the presence of factors VIIIa and X suggests that cleavage of factor IXa at Arg^{145}-Ala^{146} is essential for the exposure in factor IXa of a binding site for the factor VIIIa-factor X complex that is involved in high affinity binding of factor IXa to platelets.

The present studies that demonstrate a normal affinity of factor IXa_{CH} binding to activated platelets in the absence of factors VIII and X are in agreement with the demonstration by Jones *et al.* (15) of normal binding of factor IXa_{CH} to phospholipid (phosphatidylserine/phosphatidylcholine) vesicles by 90° light scattering. Our demonstration that factor IXa_{CH} fails to bind to activated platelets with high affinity in the presence of factors VIIIa and X is not inconsistent with the phospholipid binding studies of Jones *et al.* (15) which were not done in the presence of factors VIII and X. Finally, the present studies demonstrating abnormally low rates of factor X activation by factor IXa_{CH} are entirely consistent with the studies of Jones *et al.* (15). Thus, as shown in Table II, the catalytic efficiency (k_{cat}/K_m) calculated for factor IXa_{CH} in the presence of activated platelets and factor VIIa was 6,944 $\mu M^{-1} \cdot min^{-1}$ or 37% of normal (18,700 $\mu M^{-1} \cdot min^{-1}$) when the k_{cat} was based on the total amount of enzyme added, compared with a relative rate of factor X activation by factor IXa_{CH} shown to be 36% of normal by Jones *et al.* (15). However, when we calculated k_{cat} as moles of factor Xa formed per mol of factor IXa *bound*, the catalytic efficiency proved to be essentially normal (36,783 min^{-1} compared with 26,714 min^{-1} for normal factor IXa). This indicates that the defect in factor X activation observed with factor IXa_{CH} is attributable solely to the decreased amount and affinity of factor IXa_{CH} binding to platelets in the presence of factors VIIIa and X.

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